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The technical objectives of this project are to transfect mutant p53s into normal human mammary epithelial cells obtained from different donors and to isolate clones. The clones will be characterized for extension of lifespan and immortalization in cell culture. It will also be determined if expression of any of the mutant p53s provide a growth advantage to breast epithelial cells prior to immortalization. An additional objective is to determine in breast epithelial cells immortalized and expressing mutant p53 if expression of the mutant p53 is necessary for the maintenance of growth. Finally, downstream genomic targets of p53 that may be important in the development and progression of breast cancer will be determined.

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Introduction

Both normal human fibroblasts and epithelial cells maintained in cell culture undergo cellular senescence and do not spontaneously immortalize. A model involving a two stage mechanism for regulating cellular senescence or aging has been previously reported (Shay & Wright, 1989; Wright et al., 1989; Wright and Shay, 1992). In this model, the first stage or mortality stage 1 (M1) leads to normal cellular senescence. In order for cells to grow past this stage, the M1 mechanism must be overcome. This may be accomplished by interaction of mutant p53 with activated oncogenes such as Ha-ras (Eliyahu et al., 1984) or by the activity of DNA tumor virus gene products such as human papilloma virus type 16 E6/E7; adenovirus 5 E1A/E1B or SV40 large T antigen (Linzer & Levine, 1979; Sarnow et al., 1982; DeCaprio et al., 1988; Huange et al., 1988; Shay et al., 1989; Werness & Levine, 1990; Shay et al., 1993; Demers et al., 1994), presumably by inactivation of the tumor suppressor gene products pRb and p53. However, these DNA virus proteins fail to directly immortalize human cells. Cells overcoming M1 continue to proliferate in an "extended lifespan" period until an independent second mortality stage (M2) mechanism is activated (crisis), and only if a critical M2 gene becomes inactivated can a rare cell escape crisis and become immortal.

The p53 tumor suppressor gene product and the retinoblastoma gene product (pRb) or a retinoblastoma-like activity appear to be important in regulating the M1 stage in most human cell types (Hara et al., 1991; Shay et al., 1991; Shay et al., 1993; Gotz and Montenarh, 1995). In normal mammary epithelial cells the p53 levels remain

constant throughout their lifespan in culture in contrast to human diploid fibroblasts which showed an increase in levels of p53 as cells reached senescence (Kulju and Lehman, 1995). The pRb levels in HME cells, decrease significantly as the cells approach senescence. The p53 tumor suppressor gene is one of the most commonly mutated genes in human cancer (Harris & Hollstein, 1992), with approximately 50% of primary breast tumors containing alterations involving the p53 gene (Callahan and Campbell, 1989; Hollstein et al., 1991; Harris & Hollstein, 1992; Moll et al., 1992). Prompted by the findings of Band et al., (1990, 1991), that human mammary epithelial cells may occasionally immortalize when expressing HPV 16 or HPV 18 plasmids defective in pRb binding but normal for the E6 function of p53 abrogation, we infected human mammary epithelial cells (HME) with defective retroviruses expressing HPV 16 E6, E7 or E6/E7 (Shay et al., 1993). The results showed that HME cells expressing either HPV 16 E6/E7 or E6 alone were capable of overcoming M1 and in some instances M2. Direct support for this was recently obtained when it was demonstrated that breast epithelial cells but not breast stromal cells obtained from a patient with Li-Fraumeni Syndrome (containing a germline mutation in p53) spontaneously immortalized in cell culture (Shay et. al., 1995). That breast epithelial cells immortalize more easily than stromal cells is also supported by the epidemiological findings that the annual incidence of epithelial cell carcinomas such as breast cancer, are at least 100 times higher than the annual incidence of soft tissue sarcomas (Pollock, 1992). Tissue specific lineages are likely to have different regulatory mechanisms which may influence their ease of escape from senescence followed by immortalization. Although this does not necessarily indicate that bypassing M1 is less frequent in fibroblasts as

studies such as Bond et al., (1994) has been shown to successfully abrogate p53 function in human diploid fibroblasts with the introduction of mutant 53 143^{ala}. This is reflected in the apparently different roles that p53 and pRb have in cellular senescence for HME cells versus human mammary stromal cells (Shay et al., 1993).

Inactivation of M2 most likely involves recessive events consistent with the observations that a limited proliferative capacity is restored in hybrids between immortal and mortal cells (Pereira-Smith, 1987). Mutational inactivation of one allele followed by the elimination of the remaining wild-type allele by nondisjunctional or chromosomal conversions (Rew, 1994), selective growth advantage for the mutant (Harvey et al., 1995) or possibly telomere shortening as theorized by Wynford-Thomas et al., (1995), are all likely mechanisms for escape from M2. However, the gene product regulating the M2 stage still remains to be determined. One possibility is that it may involve a gene in the telomerase repression pathway (Shay and Werbin, 1993). The ribonucleoprotein enzyme telomerase, is involved in maintaining the stability of telomeres at the ends of chromosomes (Counter et al., 1992). The end replication problem described by Watson (1972) would lead to progressive telomere shortening in normal cells since the mechanisms of DNA replication in linear chromosomes is different for each of the two strands (e.g., leading and lagging). This progressive loss of telomeres (simple tandem repeats of the sequence TTAGGG) at the ends of human chromosomes may be a molecular mechanism that determines the time of onset of cellular senescence (Olovnikoff, 1973; Harley, 1991).

Telomerase, an enzyme expressed in germ line cells, stem cells and cancer cells (Greider and Blackburn, 1985; Greider and Blackburn, 1989; Morin, 1989;

Counter et al., 1994; Kim et al., 1994; Hiyama et al., 1995; Piatezsek et al., 1995) contains its own RNA template and thus extends the overhanging G-rich telomeric strand by direct polymerization of deoxynucleotides into tandem TTAGGG repeats. This extended G-rich strand is now used as the template for synthesizing the C-rich complementary strand. Telomerase therefore stabilizes the telomeric length in immortal and cancer cells by compensating for the end replication problem. The absence of telomerase in normal somatic cells results in loss of 50-200 bp/cell from telomeres per round of replication (Harley et al., 1990; Allsopp et al., 1992). Bypassing M1 does not reactivate telomerase and telomeres continue to shorten during the period of extended lifespan (Counter et al., 1992; Shay et al., 1993). The immortal cells that overcome M2 almost always re-express telomerase and are capable of maintaining stable telomere lengths (Counter et al., 1992; Wright and Shay, 1992; Shay et al., 1993). Escape from M2 may thus represent the abrogation in the repression pathway of telomerase activity in somatic cells (Counter et al., 1992; Wright and Shay, 1992; Shay et al., 1993; Piatyszek, 1995).

In the first year of our studies we sought to determine if the M1 mechanism in HME cells could be directly overcome by the introduction of p53 mutants implicated in a variety of human cancers. While some of the p53 mutants inserted into normal human mammary epithelial cells resulted in extension of in vitro lifespan, one clone expressing p53 mutant 273[his] immortalized.

Results

Cell culture

In culture, using defined medium supplemented with growth factors, normal human mammary epithelial cells (HME) vary in their proliferative capacities, ranging from 25-50 population doublings and then undergo morphological changes associated with finite lifespan (Shay et al., 1993; Van Der Haegen et al., 1993). Young HME cells initially display high proliferative capabilities, but as they approach M1 (the first stage of cellular senescence), the proliferation rate slows down until the cells cease to divide. Normally HME cells can remain in a senescent, growth arrested state for several months if nutrients are replaced frequently.

Transfection/infection of mutant p53 constructs

p53 "hot spot" mutants were introduced into normal HME 31 cells at PDL 28 and into HME 32 at PDL 15 either by lipofectin transfection of expression vectors, or by infection of recombinant retroviruses, containing p53 mutants. Since these cell strains were previously characterized by our laboratory for in vitro lifespan (Van Der Haegen et al., 1993), extension of lifespan is determined as a cell strain proliferating greater than 10 PDL past their oldest observed senescence point. Of 123 total clones obtained, 34 (28%) were able to bypass the M1 stage of cellular senescence and continue proliferating exhibiting an "extended lifespan" (summarized in Table 1a). Table 1b shows the range of extended lifespan for those clones able to bypass M1 and the mean PDL of extended lifespan in vitro. Lipofectin transfection yielded more clones capable of extension of lifespan than did defective retroviral infections (Figure 1a). HME 31 cells had 24% of lipofectin transfected clones with extended lifespan in

culture in comparison to 7.7% of infected clones (Figure 1a). Figure 1b summarizes

the relative efficiencies of the promoters driving the mutant p53 constructs used in this

study to overcome M1. Clones with extension of lifespan were more readily obtained

via lipofectin transfection with a CMV promoter from HME 32 compared to HME 31 and

one clone was able to bypass the M2 stage and immortalize (Figure 1c).

Table 2 summarizes the extension of lifespan in of HME 31 and 32 for each

mutant p53 introduced. HME cells were transfected with the expression plasmid

(pRc/CMV) containing the p53 mutations at either codon 143^{ala}, 175^{his}, 248^{trp} or 273^{his}

respectively, or infections with defective retroviral construct pZipneo SV(X) containing

143^{ala}, 175^{his}, 248^{trp}, or 273^{his}, respectively. The two most frequently observed p53

mutations in human cancers, e.g., 248^{arg} and 273^{his} (Hollstein et al., 1991),

demonstrated extended lifespan in all clones isolated from HME 32 and in one

instance an immortalized clone was obtained (HME 32 (273)-1). Introduction of the

expression vectors without p53 mutants did not result in extension of lifespan or

spontaneous immortalization in any instance (Table 2).

Analysis of mutant p53 proteins by Western blot

Western blot analysis of protein extracts from HME cells expressing transfected p53

mutants with monoclonal antibody PAb DO-1, revealed p53 overexpression in the 143,

175, 248 and some but not all of the 273 clones. Overexpression was not observed

however in the immortalized clone, HME 32(273)-1, nor HME 32 (273)-6, another 273

clone that did not immortalize (Figure 2a). Figure 2b shows protein levels of p53 and

pRb in young and senescent HME cells and reveals that pRb levels decrease in both senescent HME 31 and 32 cells and may explain the 100 fold greater frequency of immortalization of mammary epithelial cells versus stromal cells (Pollock, 1992). Western analysis of the MDM2 protein revealed no overexpression of this known p53 transcription target (data not shown). Experiments were then undertaken to determine if the overexpression of the temperature sensitive mutant p53 143^{ala} could repress cell growth when the wild-type conformation of p53 was expressed (32°C). While the cells continued to proliferate when the mutant conformation was expressed (37°C), the cells markedly decreased proliferation within 24 hours when shifted to 32°C (wild-type p53 conformation) in both HME 31 and 32. This was evidenced by cell counts taken at 12, 24, 36 and 48 hours after temperature shift to 32°C. For the clones containing the 143^{ala} p53 mutant, the decrease in the total cell count was 43% that of the control cells. By 48 hours in culture, cell counts were 28% that of the control counts. Three weeks in culture revealed the mean percentage of HME 31 (143)-1 and HME 32 (143)-1 cells to be 13% that of their respective controls. Thus overexpression of the wild-type p53 without DNA damage induction (Smith et al., 1995) inhibits HME growth irrespective of the age of the cells.

Analysis of the temperature sensitive mutant p53 143^{ala}

To determine relative levels of mutant 143ala p53 expression, immunoprecipitations were performed (Zhang et al., 1992). HME 32 cells expressing p53 mutant 143ala were immunoprecipitated with monoclonal antibodies PAb 240, PAb 1620 and PAb

DO-1 at both the permissive and nonpermissive temperatures (Figure 3). While a strong signal using PAb 240 was observed, detection with PAb 1620 yielded weak signal at 37°C. However, at 32°C, there was a significant decrease in the expression of mutant p53 and correspondingly, an increase in signals exhibited by PAb DO-1 and PAb 1620.

Control HME 31 and HME 32 cells were cultured at both temperatures to determine growth rates as well as to observe morphological alterations. Figure 4 shows that as expected, normal cells at 32° C grow much slower. Photomicrographs representative of the morphologies observed from these cells are illustrated in Figure 5. Normal cells at 32°C and 37°C and p53 143[ala] mutant transfected cells at 37°C share the same phenotype in culture whereas the mutant transfected cells at 32°C (wild-type conformation) become large and flat.

Analysis of immortalization of HME 32 with p53 mutant 273[his]

HME 32 transfected with the "hot spot" p53 mutant 273[his] were able to bypass both the M1 stage and after an extended crisis period also escaped from the M2 stage. Characterization of the immortalization process was followed by several techniques including terminal telomere restriction fragment (TRF) analysis, telomerase activity assays using the terminal repeat amplification protocol (TRAP), p53 DNA binding reporter assays, chromosome counts, number of population doublings in culture, as well as Western blot analysis with several p53 reactive antibodies. The presence of the original mutant p53 vector introduced was confirmed by PCR amplification of a 347

bp fragment from the CMV promoter region while the presence of the mutant within the genomic p53 gene was confirmed by SSCP (G. Tomlinson, personal communication).

The frequency of immortalization was calculated by determining the total number of cells plated and collected using what is essentially a fluctuation analysis (Shay et al., 1993). From this number, the likelihood of the single HME 32 (273)-1 clone escaping from crisis (e.g. M2) was estimated to be 2×10^{-7}. This figure is consistent with the range previously determined for human fibroblasts (Shay et al., 1993) but lower than that for HME cells expressing human papillomavirus 16 E6/E7 or SV40 T antigen (Shay et al., 1993).

Western blot analysis showed that the mutant p53 273[his] protein product was not overexpressed in the immortalized clone. Levels of p53 remained comparable at all PDL's examined (Figure 6).

In order to verify the expression of mutant p53 273[his] protein products and the lack of wild-type p53 in the immortal cells, transient transfections were performed by cotransfection of a LacZ reporter construct containing either the p53 consensus sequence or RGC consensus sequence and a luciferase reporter gene. The p53CON and ribosomal gene cluster (RGC) are DNA sequences that had been identified by their ability to bind wild-type p53 either alone (RGC) or as part of a nuclear complex (p53CON) (Kern et al., 1991; Funk et al., 1992). Chen et al. (1993) previously showed that transcriptional activation by 273[his] of the p53con sequence is comparable to wild-type activity, while little transcriptional activity is observed with the RGC consensus sequence. Figure 7 illustrates that while wild-type HME transactivation is high for both

p53CON and RGC, the immortalized HME cells containing the 273[his] mutant exhibit minimal transactivational activation of the RGC consensus but strong activity to the p53CON sequence.

PCR amplification using primers custom synthesized for the pRc/CMV p53 promoter sequence revealed the presence of the construct in the HME 32 (273)-1 clone, pRc/CMV 273 positive control, but not in the parental HME 32 cell strain (Figure 8).

TRF results are illustrated in Figure 9. Normal HME 32 have an average length of approximately 7-8 kb at population doubling 20 (PDL 20). The HME 32(273)-1 cells at PDL 60 show a significant decrease in average telomere length to approximately 3-4 kb (nearly 100 bp of the telomeres are lost per doubling). By PDL 80 there is no further shortening and telomere length has stabilized at about 2kb. Additional TRF analysis at PDL 225 showed no change in the telomere length from that observed at PDL 80 and 100 (data not shown).

A PCR based assay for the measurement of telomerase activity (Kim et al., 1994; Piatyszek et al., 1995) was performed (Figure 10). The parent cells at PDL 20, and precrisis HME 32(273)-1 cells PDL 41 had no detectable telomerase activity while from PDL 60 on, HME 32 (273)-1 did. This is consistent with a rare telomerase positive clonal population emerging around PDL 50-60 and quickly predominating the population of cells which were initially heterogeneous with a population of normal cells undergoing crisis and continuing to shorten their telomeres. Co-amplification of a genomic internal DNA standard insured the lack of Taq polymerase inhibitors which could yield false negatives. Quantitation against this standard also revealed that there

was no significant increase in the activity of telomerase with increasing PDL.

Metaphase spreads were counted on HME 32(273)-1 cells after 150 PDLs in culture. Analysis consisted of counting 27 random metaphase spreads. Of the spreads counted, a range of 62-139 chromosomes was observed. The median chromosome spread value was 81. Eleven percent of the population was pseudotetraploid, 22% were triploid, with the remaining 67% aneuploid.

Materials and Methods

Cell lines and cell culture

Normal mammary epithelial cells (HME) 31 were obtained from a patient undergoing breast cancer surgery. HME 32 was obtained from a patient undergoing reduction mammoplasty. Cells were cultured as previously described (Van Der Haegen et al., 1992). Briefly, serum-free medium was utilized, consisting of a modified basal medium MCDB 170 (MEBM, Clonetics, San Diego, CA,) supplemented with 0.4% bovine pituitary extract (Hammond Cell Tech, Alameda, CA,); 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 25 μg/ml gentamicin, 10 ng/ml transferrin (Sigma, St. Louis, MO,); and 10 ng/ml epidermal growth factor, (Collaborative Research, Bedford, MA,). Medium was changed every other day. Primary cultures were originated from organoids and grew predominantly as epithelioid cell populations. Following a 'self-selection' process HME 31 could be subcultured for an additional 45 - 50 doublings while HME 32 could only be subcultureed for an additional 25 - 30 doublings. These cell strains

have been previously characterized by our laboratory (Van Der Haegen et al., 1993).

Recombinant retroviral vectors

Retroviral vectors consisted of the parent vector pZipNeoSV(X) or pZipNeoSV(X) containing the genes for 143[ala] mutant p53; 175[his] mutant p53; 248[trp] mutant p53; 273[his] mutant p53 under the transcriptional regulation of the Moloney murine leukemia virus promoter-enhancer sequences (MLVLTR). These vectors also contained the gene conferring neomycin resistance under the transcriptional regulation of the SV40 promoter. The mutant p53 vectors were all kindly provided by Curtis Harris (National Cancer Institute, Bethesda, MD). Recombinant viruses were generated in the amphotrophic packaging line PA317 according to previously described procedures (Shay et al., 1993). Viruses produced from the PA317 cells were used to infect HME cell strains 31 at PDL 22 and 32 at PDL 18, similar to those described by Halbert et al., (1991;1992). The cells were selected on G418 (50-100 µg/ml) and clones isolated. Each HME clone was subcultured and continuously passaged at 2×10^5 cells/T75 flask and tested for escape from crisis at a total population size of $1\text{-}2 \times 10^6$.

Transfections

HME 31 and 32 cells were Lipofectin (Gibco/BRL, Gaithersburg, MD) transfected at the same aforementioned PDLs with pRc/CMV constructs containing single p53 genes mutated at codons 143[ala], 175[his], 248[trp] and 273[his] respectively. These pCMV-Neo-Bam constructs were kindly provided by Dr. John Minna (Simmons Cancer Center, UT Southwestern Medical Center, Dallas, TX). The neomycin resistant gene was included as a dominant selectable marker. The transfections were performed under the

following conditions optimized for HME cells; 10-20 µg of plasmid DNA was incubated with 150 -200 µl of Lipofectin at room temperature for 15-45 min. This newly formed complex was then added to 4 ml of Opti-MEM II transfection medium (Gibco/BRL, Gaithersburg, MD) and incubated at 37°C for 6-10 h. After incubation this medium was removed and replaced with fresh MEBM. Cells were allowed to recover for 24 - 48 h, then trypsinized, counted, replated at various densities with G418 added. After approximately 2 weeks,individual colonies were ring clone isolated and cultured as described above.

Transient Transactivation Analysis

Transient transfections were performed by electroporation with a Gene Pulser apparatus (Bio-Rad, Hercules, CA.) as previously described (Chen et al., 1993). At 70-80% confluency HME 32 and HME 32(273)-1 cells approximately 2×10^7 cells (0.5 ml) were mixed with 60 µg of DNA (20 µl) containing 10 µg pCMV-LacZ expression plasmid plus 10 µg of reporter plasmid supplemented with sonicated salmon sperm DNA. Cells were incubated at room temperature for 5 min followed by electroporation at 960 µF, 240 and 260 volts. Transfected cells were immediately re-suspended with pre-warmed medium and incubated under normal conditions. Within 12-24 hours, the medium was changed. Forty-eight to 72 hours post-transfection, cell extracts were prepared and luciferase activity measured as previously described (Funk et al., 1992). Luciferase activity was assessed and normalized for differences in transfection efficiency as determined by a spectrophotometric ß-galactosidase assay

Gel electrophoresis and immunoblotting

Cell extracts were prepared as per Gillespie and Hudspeth (1991), using a modified

buffer lacking ß-mercaptoethanol until time of denaturation. Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL). Samples were separated on 10% SDS-PAGE gels with 4% stacking gels using a mini-gel apparatus (Bio-Rad Mini Protean II System, Richmond, CA).

Immunoblotting, incubation and developing procedures followed the protocol for chemiluminescence detection of proteins as modified by Gillespie and Hudspeth (1991). Briefly, after electrophoresis, proteins were transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL) and incubated with a monoclonal, primary antibody [either anti-p53 clone Pab 1801 or DO-1 recognizing wild-type and mutant conformations; (Oncogene Science, Cambridge, MA)], followed by a goat-antimouse IgG secondary antibody conjugated to alkaline phosphatase. pRb was detected using MAb-1 for the pRb gene product (Triton Diagnostics, Alameda, CA). Chemiluminescent reagents, nitro-block and CSPD (Tropix, Bedford, MA), were used for signal detection and blots were exposed on Fuji X-ray film.

PCR amplification of pRc/CMV

The conditions for PCR amplification of sequences from within the CMV promoter of pRc/CMV are as follows: primers; 5'-ATAGTAATCAATTACGGGGTCATT-3', 5'-TATCGCTACTGATTATGCATCTAC-3'. These primers amplify a 347 base pair fragment which contains codons 260 → 607. Cocktail for the 50µl reaction contained 1.5mM $MgCl_2$, 2.5U of Taq polymerase (Gibco/BRL), 200mM dNTP's, 50pM of primers, 100 ng genomic DNA from HME 32 cells at PDL 18 or HME 32 (273)-1 cells at PDL 225, 20 ng plasmid DNA. PCR program was as follows: 94°C, 1 min; (94°C, 30s;

55°C, 30s; 72°C, 30s) x 40; 72°C, 1min. The PCR product was then run on an 0.8% agarose gel for 45 min. at 85V, stained with 0.05 µg/ml ethidium bromide and photographed.

Telomere length measurements

DNA from normal HME 32 and HME 32(273)-1 immortalized cells at PDLs 60, 80 and 100 was digested with Hinf1. 10 µg of each sample was run on a 0.8% agarose gel overnight at 70 volts constant. Gels were dried under vacuum at 50°C for 45 min, soaked in 0.5M sodium hydroxide/1.5M sodium chloride for 15 min, neutralized in 0.5M Tris pH 8.0/1.5M sodium chloride for 15 min. Gels were then prehybridized in 5X SSC (standard saline/citrate), 5X Denhardt's, 0.5mM sodium pyrophosphate, 10mM disodium hydrogen phosphate at 37°C for 4-6 h. A second incubation in fresh solution with the ^{32}P end-labeled telomeric probe (TTAGGG)$_4$ for 12h followed. After washing three times in 0.1X SSC at room temperature (7 min each), the gel was either exposed to X-ray film or analyzed on a phosphorimaging device (PhosphorImager, Molecular Dynamics, Sunnyvale, CA, USA).

Telomerase assays

A one tube PCR-based telomerase assay was performed as originally described (Kim et al., 1994) with some modifications (Wright et al., 1995). The assay was performed in two steps: 1) Telomerase mediated extension of an oligonucleotide primer (TS), which serves as a substrate for telomerase; and 2) PCR amplification of telomerase activity product (an incremental 6 nt ssDNA ladder) with the oligonucleotide primer CX in a competitive amplification reaction with a 150 bp fragment encoding aa 97 - 132 of rat

myogenin as an internal telomerase amplification standard (ITAS).

Details of the method are as follows: For the cells in culture, 10^5 cells were pelleted in culture medium. The supernatant was removed and the dry pellet stored at -80°C. The cells were lysed with 200 µl of ice cold lysis buffer consisting of 0.5% CHAPS, 10 mM Tris-HCl [pH 7.5], 1 mM MgCl2, 1 mM EGTA, 10% glycerol, 5 mM beta-mercaptoethanol, 0.1 mM AEBSF and left on ice for 30 min. The lysate was centrifuged at 14,000 RPM for 20 min at 4°C, and 160 µl of supernatant was collected into an Eppendorf tube making sure that no traces of the pellet were withdrawn; flash-frozen in an EtOH-dry ice bath and then stored at -80°C. Generally 2 µl of each lysate was analyzed containing the equivalent of approximately 1000 cells. In some instances the concentration of the protein in the extract was measured using the BCA protein assay kit (Pierce Chemical Company, Rockford, IL) and an aliquot of the extract containing approximately 6 µg of protein used for each telomerase assay.

Specificity of the processive 6 nt ladder is demonstrated by RNAse treatment. For RNAse controls, 5 µl of extract is incubated in 1 µg of RNAse (5'-3', Boulder, CO) for 20 min at 37°C. A 2 µl aliquot of extract is then assayed in 50 ul of reaction mixture containing 50 µM each dNTP, 344 nM of TS primer (5'-AATCCGTCGAGCAGAGTT-3'), and 0.5 µM of T4 gene 32 protein (USB, Cleveland, OH), [α^{32}P]dCTP, 5 attograms ITAS and 2 units of Taq polymerase (Gibco/BRL, Gaithersburg, MD) in a 0.5 ml tube which contained the CX primer (5'-CCCTTACCCTTACCCTTACCCTAA-3') at the bottom sequestered by a wax barrier (Ampliwax^{TM}, Perkin-Elmer, Foster City, CA). After 30 min of incubation at room temperature for telomerase mediated extension of

the TS primer, the reaction mixture is heated to 90°C for 90 seconds for inactivation of telomerase, and then subjected to 31 PCR cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 45 seconds. The PCR products were electrophoresed on a 10% acrylamide gel as previously described (Kim et. al., 1994). Since human telomerase is processive, during the initial 30 minutes of incubation, in the presence of the TS primer, varying numbers of hexameric repeats are added to the primer and when subsequently amplified yield a 6-bp DNA incremental ladder. Extracts from the samples not containing telomerase do not extend the TS primer (Kim et al., 1994).

Metaphase spread analysis

Methods for obtaining metaphase spreads were described previously (Aldaz et al., 1989). Cultures were incubated with 0.01μg/ml Colcemid (Gibco/BRL, Gaithersburg, MD) in fresh medium for 4 h. After collection by trypsinization, cells were incubated for 1 h at 37°C in a 0.067M KCl hypotonic solution then fixed in 3:1 methanol:glacial acetic acid, rinsed and spun 2X for 5 min each at 1200 RPM . After resuspension in 1-2ml of fix, pellets were dropped onto precleaned microscope slides and stained with Giemsa Stain (Sigma Chemical Company, St. Louis, MO). Chromosomes were counted from >25 randomly chosen metaphase spreads.

Fluctuation analysis

The frequency of escape from crisis (e.g. immortalization frequency) was estimated using an approach based on what is essentially a fluctuation analysis previously described (Shay et al., 1993). Clones were expanded several population doublings before crisis into multiple series in several sizes of culture dishes at a constant cell density. Each series was subsequently maintained as a separate culture, so that at the

end of the experiment the fraction of each series that gave rise to an immortal cell line could be determined. Using different size dishes permitted series to be set up which contained a different number of cells per dish while maintaining a constant culture environment (cells/cm^2). Cultures were split at or just prior to confluence. Once cells reached crisis, they were split (at least once every 3 weeks) until only a few surviving cells remained or the culture had immortalized. Immortalization was expressed as the number of immortal lines per number of culture series. Frequency is expressed as the probability of obtaining an immortal cell line based on the total number of independent immortalization events and dividing by the total number of cells plated.

Conclusions

The onset of breast cancer is generally associated with increased aging except in cases of Li-Fraumeni Syndrome and familial breast cancers (Li et al., 1988; Malkin et al., 1990; Kessler, 1992; Smith et al., 1992; Malkin, 1993). Previously, it has been reported that abrogation of normal p53 function may be important in the immortalization of HME cells (Band et. al., 1991; Shay et. al., 1993). The results of the first year's studies illustrate that some but not all p53 mutants can neutralize the M1 mechanism of cellular senescence in human mammary epithelial cells (HME) in culture. Previous studies have shown that for most human cells to abrogate the M1 stage of cellular senescence both p53 and an Rb-like function must be overcome (Munger et al., 1989; Woodworth et al., 1989; Watanabe et al., 1989; Band et al.,1990). While HPV16 E6 only gives partial extension of lifespan in IMR90 fibroblasts as does HPV16 E7, combinations of the two result in the full extension lifespan (Shay et al.,

1993). Thus, for cells to reach the M2 stage and have the potential to immortalize, the cellular mechanisms involved in the M1 mechanism must be fully overcome. It has been shown that for HME cells, abrogation of only p53 is required to fully overcome the M1 stage. HPV16 E6 results in most HME cells reaching M2 and then a relatively rare cell in the population immortalizes (Shay et al, 1993). In addition, we recently reported the spontaneous immortalization of HME from a patient with Li- Fraumeni Syndrome (germline mutation in p53). Wazer et al. (1995) recently showed immortalization of distinct mammary epithelial cell types by HPV 16 E6 or E7 while Tsutsui et al. (1995) demonstrated extension of in vitro lifespan with the carcinogen Aflatoxin B1. Our work during the first year of this project extends these observations further to demonstrate that expression of mutant p53 into normal HME can also result in immortalization. The clone HME 32 (273)-1 was observed to have telomeres that continued to shorten with progressive subculturing as well as the reactivation of telomerase upon reaching M2. PCR revealed the presence of the construct within the cells, SSCP showed the presence of the mutant allele (G. Tomlinson, personal communication) while continuous subculturing has the cells at PDL 300. These observations taken together provide evidence for the immortalization of HME 32 by p53 mutant 273[his].

We observed different relative efficiencies of expression vectors carrying mutant p53 to extend lifespan of the HME cells. The CMV expression vectors containing p53 mutants were more efficient in extending lifespan and abrogating wild-type p53 function (as indicated by immunoblotting and immunoprecipitations) but less so than HPV16 E6 (which degrades p53) in which over 90% of the clones had extension of

lifespan (Shay et al., 1993). The p53 mutants carried in the defective retroviral vectors were not only less effective in inducing extension of lifespan but also did not give rise to any immortal clones. Thus, sequestration or degradation of a critical amount, if not all, of wild-type p53, appears to be required for HME cells to completely overcome M1, thereby fully extend cellular lifespan, and reaching the M2 stage. For some of the p53 mutants, M1 was not completely bypassed and thus, no immortalization was observed. One explanation is that the relative strength of the promoters in the expression vectors used to carry the mutant p53 to the target cells may be important in determining the amount of wild-type p53 neutralized. Liu et al., (1995) showed that the oncogenic potential of the HPV 16 E7 protein is regulated by the level of E7 expression which in turn is dependent upon the promoter driving expression. Milner and Medcalf (1991) showed that p53 dominant negative mutant expression can overcome the wild-type. Wyllie et al., (1995) reported complete abrogation of p53 function in thyroid cell lines whereas Williams et al., (1995) showed incomplete abrogation of wild-type p53 via dominant - negative effects for 4 mutant p53s introduced. Bond et al. (1994) showed the abrogation of M1 in normal diploid human fibroblasts using a retrovirally packaged 143^{ala} mutant. Our studies indicate that a required amount of p53 may need to be inactivated in order to obtain an extended lifespan. Another possible explanation may be that the manner in which p53 is abrogated is important. HPV16 E6, a viral oncoprotein, degrades the majority of p53 protein product via the ubiquitin pathway (Scheffner et al., 1990). It is possible that HPV16 E6 may also have other functions besides binding p53 which could contribute in a small way to overcoming M1 completely. CMV expression vectors are considered strong promoters due to the

characteristic of having better and/or more binding sites for transcription factors Liu et al., (1995). Therefore, while the mutant p53 transfected into the cells may sequester the wild-type p53 product very quickly, there may be heterodimerization occurring which protects some of the wild-type p53 in the cell. This may explain why there is a lower frequency of extension of lifespan and immortalization compared to HPV16 E6. In contrast, p53 mutants in retroviral vectors such as pZipneo may not bind sufficient wild-type p53 product to fully extend lifespan. The present results seems to support this explanation in that those cells that were transfected with mutant p53 in the context of pCMV-Neo-Bam construct resulted in a higher frequency of extension of lifespan. (13 of 52 clones, 25%) versus the recombinant retroviral constructs pZIPNeoSV(X) carrying the mutant p53 genes (4 of 48 clones, 8%).

In addition to the relative strength of the expression vectors and the amount of wild-type p53 function neutralized, factors such as the specific mutant p53 inserted into the cells and the genetics of the strain used may also be important. Cho et al., (1994) identified two major classes of p53 mutants. The first class consists of residues that interact with DNA. Missense mutations at these sites would abrogate p53 by eliminating critical DNA contacts. 273^{his} and 248^{trp}, two major "hotspot" mutations in human cancer, are examples of this class. The second class of p53 mutants exhibits abnormal structure due to missense mutations at sites crucial to the conformational architecture of the core domain (Cho et al., 1994). These "structural" mutants abrogate DNA binding through their effect on the tertiary structure (Milner, 1995). Therefore, different p53 mutants may be more effective in abrogating wild-type p53 than others. In our studies conducted during the first year of this fellowship, we found that the

transfected CMV-p53 273[his] mutant was the most efficient at ablation of p53 function to bypass M1 for in vitro extended lifespan.

Another factor which may be important is the recipient cell type and strain used. Milner and Medcalf (1991) indicated that preliminary screening of the cell lines is necessary since the internal milieu of the cell may also affect the conformation of p53. Finlay et al., (1989) showed that the phenotype of mutant p53 can be determined by the cell line used for target transfection of the mutant p53 allele. Forrester et al., (1995) reported that the effects of p53 mutants on wild-type p53 mediated transactivation are cell type dependent and that there is an enhanced activity with 273[his] on wild-type p53. In the present series of experiments it appeared that HME 31 and HME 32 demonstrated differences in their receptiveness to both particular mutants and mechanisms of introduction of mutant p53. For example, using HME 32, extension of lifespan via transfection was observed, whereas no clones exhibited extension of lifespan by infection. HME 31 however, appeared to be equally receptive for either method in extending in vitro lifespan. Both HME 31 and 32 have been previously reported to contain wild-type p53 (Shay et al., 1993).

It has been previously reported (Shay et al., 1992; Shay et al., 1993) that the clones generally emerging from M2 and continuing to proliferate indefinitely are those that maintain a chromosome complement near diploid. Cell hybrid studies have indicated that overcoming the M2 mechanism is likely to be a recessive event and thus requires the loss of two alleles. Experimentally, only a small fraction of fibroblast clones expressing T-antigen or HPV16 E6/E7 ever immortalize whereas a slightly higher fraction of HME cells do. We proposed that since a greater percentage of

fibroblasts tend to become tetraploid upon expression of T-antigen or HPV16 E6/E7, cells would have to eliminate/alter/mutate several additional alleles of a critical gene regulating the M2 stage prior to immortalization. However, HME cells expressing T-antigen or HPV16 E6/E7 or E6 alone maintain a population of both pseudodiploid and pseudotetraploid cells within most clones and the cells that eventually overcome the M2 stage and continue proliferating almost always have a near diploid chromosome constellation (Shay et al., 1993). In contrast, metaphase spread analysis of the immortal clone HME 32 (273)-1, showed a mean chromosome number of 81 with a range from 67-139. Eyfjord et al., (1995) showed that p53 mutations lead to genomic instability in primary breast tumor cells. Since this immortalized cell line appears to be established through a dominant-negative p53 mediated event, perhaps a similar mechanism is occurring, one which may be related to the loss/mutation of p53 and/or particular alleles carrying putative tumor suppressor genes with selection for the mutated allele. This may indicate that while HPV 16 degrades p53, there are no missense or other mutations present. Therefore direct mutations of p53 may play a role in genomic instability. The question of nonrandom chromosomal event(s) in this cell line is now being addressed.

In conclusion, a better understanding of the molecular events required for normal human cells to escape the protective blocks against a critical step in the development of cancer (e.g. immortalization) are beginning to emerge. Cells have to overcome two independent cellular protective mechanisms (M1 and M2) to become immortal. While alterations/mutations in p53 appear to be important in overcoming the first block, little

is presently known about the second block although there is a strong correlation with re-expression of telomerase indicating that the M2 mechanism may be associated with the regulatory pathway in the repression of telomerase activity. In addition, we have observed that if the M1 mechanism is not completely overcome or neutralized then the cells only partially escape the M1 blockade and do not appear to reach M2 thus, decreasing the likelihood of cellular immortalization. These results help explain why differing methods used to abrogate wild-type p53 function are not equally efficient at immortalizing cells and reaffirms that the "guardian of the cell", p53 (Lane, 1992), is a formidable protective protein. For example, individuals inheriting germline mutations in one p53 allele do not generally develop cancer for several decades. In addition, breast epithelial cells from patients with Li-Fraumeni syndrome require extensive in vitro culture prior to spontaneously immortalizing (Shay et al., 1995). These results indicate that perhaps other events such as loss of karyotypic stability and the genetic background of the tissue and cell type appear also to be important in the loss of the remaining wild-type p53 allele eventually resulting in the escape from M2. Our results obtained during the first year's studies provide a clearer understanding of the molecular events required for immortalization.

In summary, we have made important progress on tasks 1 and 2 of the original proposal and have initiated experiments to accomplish task 3. During the next review period we will continue our experiments on tasks 1-3 and initiate experiments to accomplish task 4. Time permitting, we will also initiate experiments to accomplish task 5.

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Table 1a Clones obtained and percent extension of *in vitro* lifespan for mutant p53s introduced into HME 31 and HME 32

Cell strain	Lipofectin transfections: no. clones with extended lifespan/total no. clones	Retroviral infections: no. clones with extended lifespan/total no. clones
HME 31	11/45 (24%)	4/52 (7.7%)
HME 32	18/23 (78%)	1/3 (33
Total % extended life	29/68 (43%)	5/55 (9%)

The total number of clones obtained from both methods used to introduce mutant p53 constructs into normal HME cell strains in this study. Lipofectin transfections plasmid construct products were under the control of the CMV promoter. Defective retroviral infection plasmid products were controlled by the Moloney MLV-LTR. CMV=cytomegalovirus; MLV-LTR=murine leukemia virus, long term repeat.

Table 1b Mean and range of the extended lifespan *in vitro* for those clones with mutant p53 which bypassed M1

Mutant p53s and vectors	Cell strain	No. of clones	Range of lifespan (PDL)	Extended lifespan (PDL)	Mean lifespan (PDL)
vector - pCMV	HME 31	4	46.3 - 51.2	none	48
143ala pCMV	HME 31	ND	ND	ND	ND
175his pCMV	HME 31	7	62.5 - 67.3	17.3	64.2
248trp pCMV	HME 31	1	63.1	13.1	63.1
273his pCMV	HME 31	3	61.6 - 72.7	22.7	68.8
pZipNeoSV(X)	HME 31	6	47.2 - 50.2	none	49.3
143ala pZipneo	HME 31	2	65.7 - 70.2	20.2	68.0
175his pZipneo	HME 31	0	none	none	none
248trp pZipNeo	HME 31	2	63.3 - 67.3	17.3	65.3
273his pZipNeo	HME 31	0	none	none	none
vector - pCMV	HME 32	3	24.6 - 28.3	none	26.1
143ala pCMV	HME 32	3	42.0 - 55.1	22.1	48.3
173his pCMV	HME 32	1	44.6	14.6	44.6
248trp pCMV	HME 32	6	44.1 - 58.2	28.2	47.5
273his pCMV	HME 32	8	42.7 - 59*	29.4*	54.6*
pZipNeoSV(X)	HME 32	2	28.6 - 28.7	none	28.65
143ala pZipNeo	HME 32	1	43.3	13.3	43.3

ND = not none
* The immortal clone HME 32 (273)-1 was not used in calculating these averages.

Table 2 Clones obtained for each mutant p53 introduced into HME 31 and HME 32

	CMV 143	CMV 175	CMV 248	CMV 273	MLV 143	MLV 175	MLV 248	MLV 273	2neo	pZipNeo SV(X)
HME 31	ND	7/30 23%	1/7 14%	3/8 38%	3/12 25%	0/10	2/9 22%	0/2	0/4	0/6
HME 32	3/6 50%	1/3 33%	6/6 100%	8/8 100%	1/3 33%	ND	ND	ND	0/4	0/6

Comparison by percentage of the individual p53 mutants introduced and the number of clones obtained that were able to bypass M1 and achieve extended lifespan *in vitro* . Mutants: 143ala; 175his; 248trp; 273his. 2neo and pZipNeoSV(X) were control vectors tested for the transfections and infections respectively. ND = not done

Figure legends

Figure 1. (a) The relative efficiency of the promoters used to introduce mutant p53 into the cells in this study to bypass M1 and induce extension of lifespan. CMV:cytomegalovirus promoter; MLV:Moloney murine leukemia virus promoter-enhancer sequences. (b) Comparison of the p53 mutants introduced via each vector and their ability to extend lifespan in culture. p53 mutants are: 175^{his}, 248^{trp}, 273^{his}. (c) Variability between cell strains is illustrated by comparing the efficiency of bypassing M1 and extending in vitro lifespan.

Figure 2. (a) p53 expression levels are illustrated from representative p53 mutant clones. Each lane contains 40 µg of protein and p53 was detected using anti-p53 monoclonal antibody PAb 1801. All clones shown, were lipofectin transfected for the p53 mutants. (b) Relative levels of endogenous p53 and pRb are shown for young and senescent normal HME. p53 levels were detected using PAb 1801. Monoclonal antibody MAb-1 was used to detect levels of pRb.

Figure 3. Immunoprecipitation results for the p53 conformation of the p53 temperature sensitive mutant 143^{ala}. p53 was immunoprecipitated with the indicated antibodies at 37°C and 32°C from HME 32 cells transfected with the 143^{ala} p53 mutant as described in the Methods section. An antibody against c-myc was used as a negative control. KDa: molecular weight protein markers sizes.

Figure 4. Growth of HME at 32°C and 37°C. Cultures at 32°C were approximately 3-4 PDL slower than those at 37°C. Proliferation was markedly decreased by 24 hours in culture after temperature shift to 32°C. By 3 weeks, cell counts in the transfected cells were 10% that of the control cells.

Figure5. Photomicrographs representative of temperature effects on the conformation of the p53 mutant 143^{ala} introduced into HME cells. Morphologically there was no difference in cell appearance for control and 143^{ala} expressing cells at 37°C nor for the control cells at 32°C. At 32°C however, there was a marked change in the morphology toward a senescent-like phenotype. At 37°C, 143^{ala} has a mutant conformation and no DNA transactivation activity. At 32°C the mutant exists in a wild-type conformation, resulting in overexpression of wild-type p53, cell enlargement and inhibition of cell proliferation.

Figure 6. Western blot analysis of the p53 expression levels for HME 32 and HME 32 (273)-1 at different times in culture. Extracts were detected using the PAb DO-1 antibody (20 μg were loaded per lane). No significant increase in p53 expression was observed.

Figure 7. Luciferase activity analysis. The transactivation activity of HME 32 and HME 32 (273)-1 was standardized against ß-galactosidase activity electroporated into both parental cells HME 32 and the clone HME 32 (273)-1. The transactivation activity

refers to the luciferase activity as measured against basal ß-gal activity in the control cells. Immortal cells were tested at PDL 250, normal cells at PDL 18.

Figure 8. Polymerase chain reaction analysis to detect the presence of the pRc/CMV construct within the immortalized clone. DNA from the originally transfected construct was used as a positive control. Normal HME 32 cells were tested at PDL 18. As shown, the presence of the construct is confirmed in the clone but not the parental cell strain. A 347bp fragment of the CMV promoter region is the product from the PCR reaction.

Figure 9. TRF analysis by Southern blot. Immortal cells at PDL 60, 80 and 100 were analyzed for decreasing telomere length vs normal, parent cells at PDL 23. A 24-mer, $(TTAGGG)_4$, was used as a probe. Telomeres stabilized to approximately 2.5 kb in length.

Figure 10. Telomerase activity analyzed using the telomeric repeat amplification protocol (TPAP). Parental cells (PDL 20) and precrisis cells had no detectable telomerase activity. Telomerase activity was first observed at about PDL 60 with no subsequent significant increase in telomerase activity with increasing passage as measured against the internal telomerase amplification standard (ITAS) as described in the Methods. Controls consisted of lysis buffer, dilutions of 10 cell, 100 cell and 1000 cell equivalents of a telomerase expressing cell line immortalized with HPV 16 E6/E7 mixed with normal cells from the original parent strain.
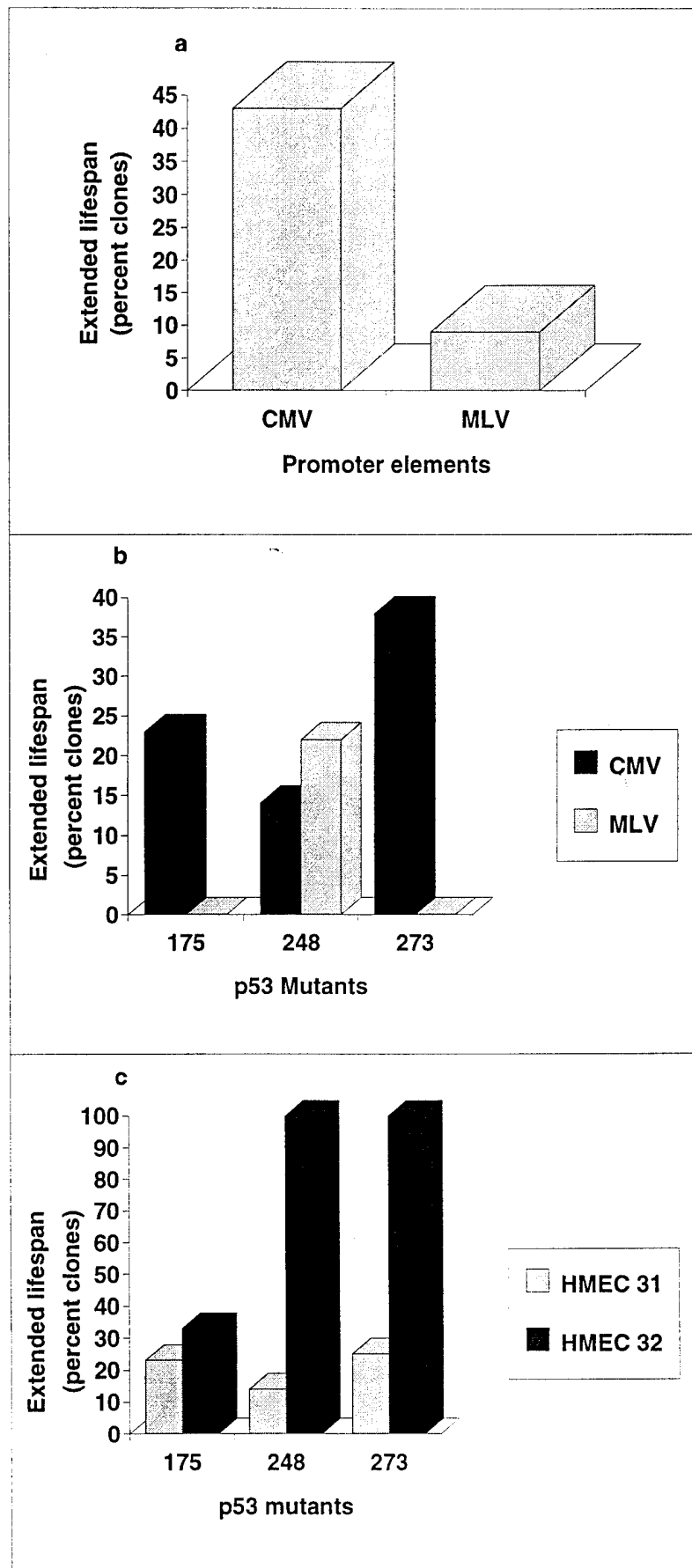
figure 1

a.

HME 32
HME 32 (273) -1
HME 32 (248) -4
HME 32 (273) -6
HME 31
HME 31 (ZIPneo) -3
HME 31 (143) -1
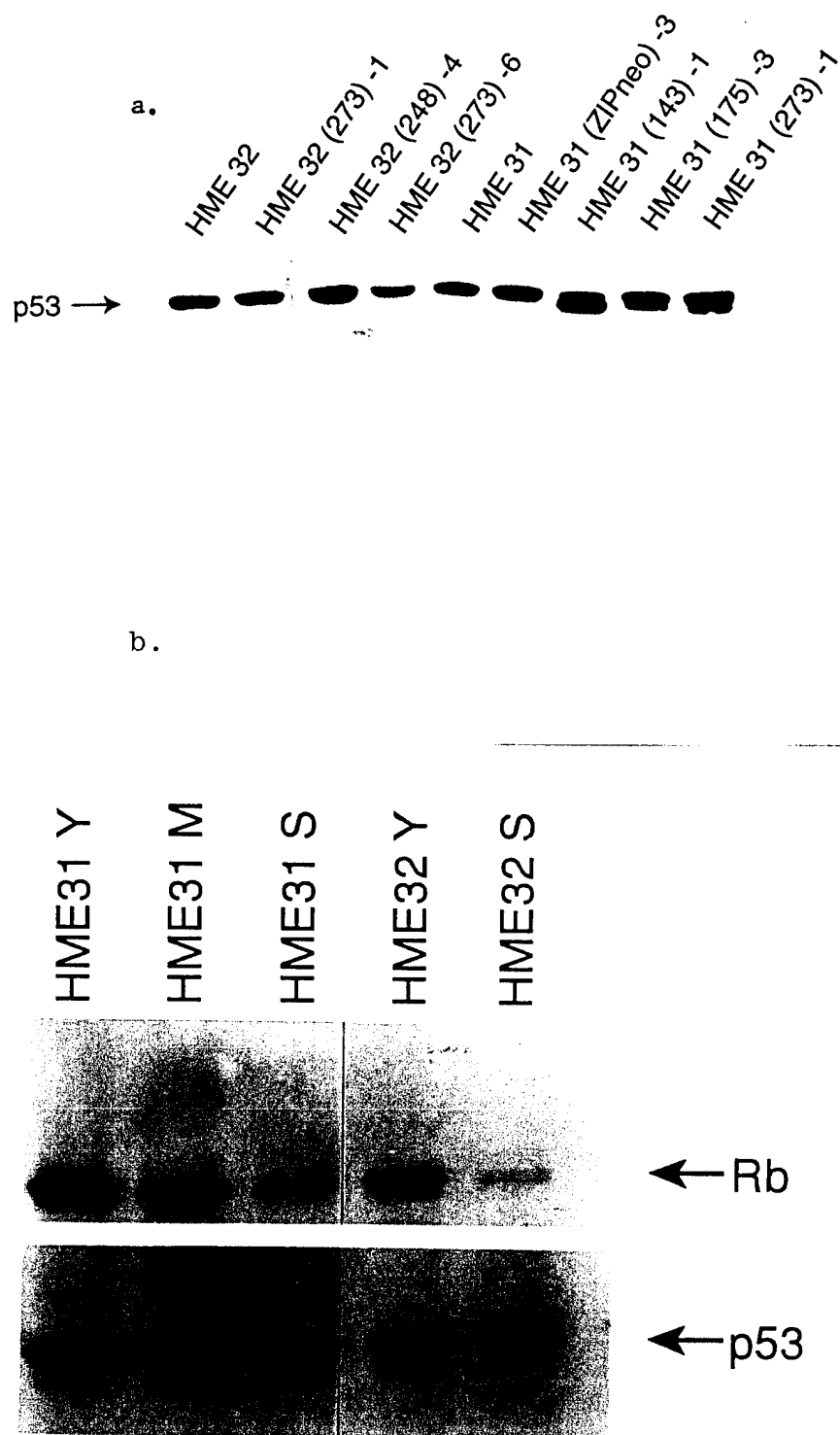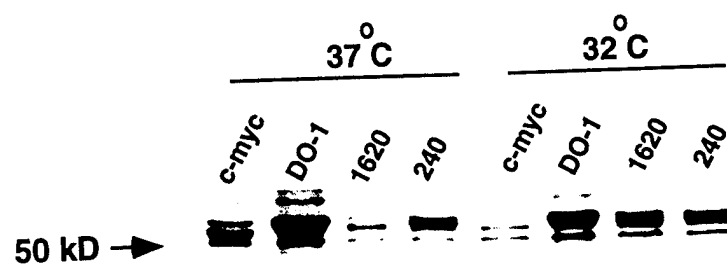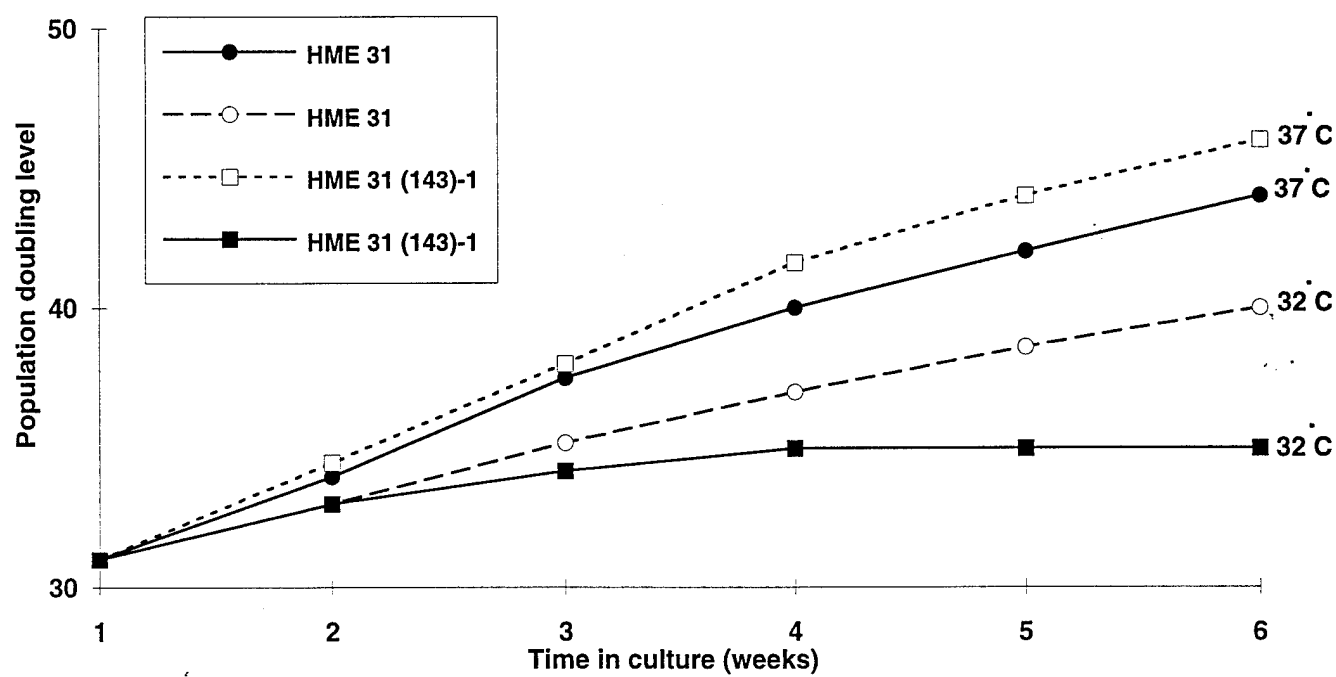HME 31 (175) -3
HME 31 (273) -1

p53 →

b.

HME31 Y
HME31 M
HME31 S
HME32 Y
HME32 S

← Rb

← p53

Figure 2.

Figure 3

figure 4
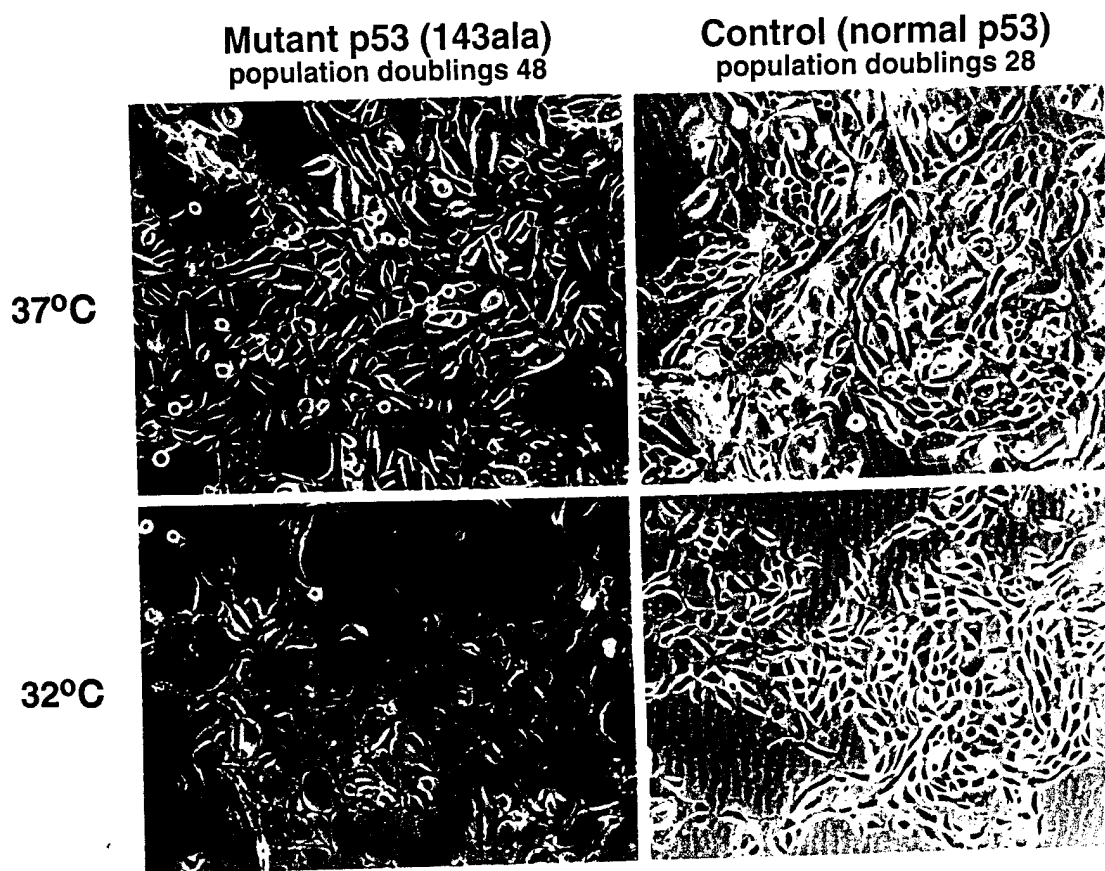
Figure 5.
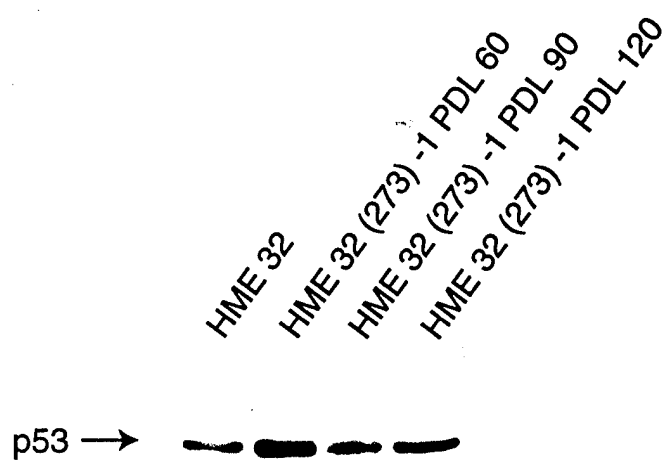
Figure 6

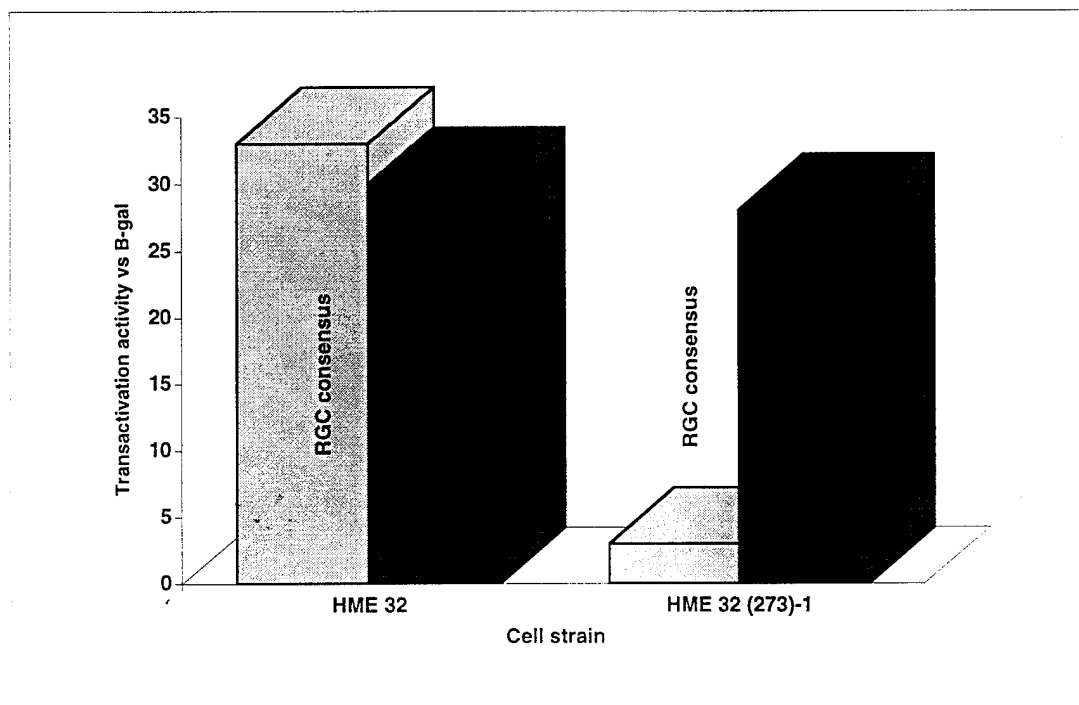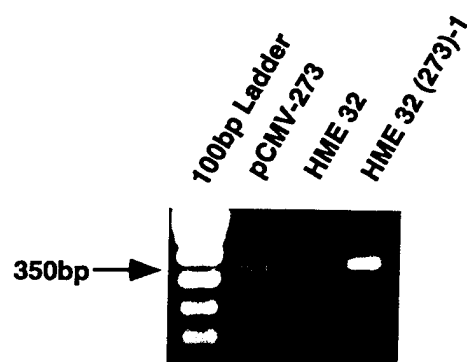|	HME 32	HME 32 (273)-1
RGC	33	3
p53	30	28

Figure 7

Figure 8

Figure 9

Figure 10